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Arabidopsis *TCH4*, Regulated by Hormones and the Environment, Encodes a Xyloglucan Endotransglycosylase

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Adaptation of plants to environmental conditions requires that sensing of external stimuli be linked to mechanisms of morphogenesis. The Arabidopsis *TCH* (for touch) genes are rapidly upregulated in expression in response to environmental stimuli, but a connection between this molecular response and developmental alterations has not been established. We identified TCH4 as a xyloglucan endotransglycosylase by sequence similarity and enzyme activity. Xyloglucan endotransglycosylases most likely modify cell walls, a fundamental determinant of plant form. We determined that *TCH4* expression is regulated by auxin and brassinosteroids, by environmental stimuli, and during development, by a 1-kb region. Expression was restricted to expanding tissues and organs that undergo cell wall modification. Regulation of genes encoding cell wall–modifying enzymes, such as *TCH4*, may underlie plant morphogenetic responses to the environment.

INTRODUCTION

Plant development is unusual because it is plastic and can be influenced dramatically by environmental conditions. For example, plants exposed to mechanical stimuli, such as wind or touch, undergo alterations in development, called thigmomorphogenesis. These alterations generally include a decrease in longitudinal growth, an increase in radial expansion, and changes in flexibility and/or strength (reviewed in Biddington, 1986; Jaffe and Forbes, 1993; Mitchell and Myers, 1995). These changes are likely adaptive because thigmomorphogenetic plants are often more resistant to additional mechanical perturbations. Such developmental plasticity is probably especially important for nonmotile creatures that are unable to escape or seek shelter from environmental stresses and instead must acclimate to the local environment.

How plants sense and respond to the environment is therefore fundamental as well as undoubtedly complex. Exogenous signals may act through endogenous growth regulators, such as hormones, or through separate transduction pathways that must run coordinately with morphogenetic processes. The molecular, enzymatic, and cellular events that act to generate the plant form and the environmental regulation of these processes are not known. However, it is clear that the placement, magnitude, and orientation of wall extensibility as well as structural characteristics of the cell wall are critical in determining plant form, size, and mechanical properties (McNeil et al., 1984; Varner and Lin, 1989; Carpita and Gibeaut, 1993). Thus, to alter plant morphogenesis during adaptation to environmental conditions undoubtedly requires cell wall modification.

The primary plant cell wall is composed of cellulose microfibrils embedded in a matrix of hemicellulose (heterogeneous and usually branched polysaccharides), pectin, and proteins (reviewed in Carpita and Gibeaut, 1993). The major hemicellulose in dicots is xyloglucan. Xyloglucans consist of (1-4)-β-linked D-glucosyl residues of which ~75% have single xylosyl residue side chains or trisaccharides of xylosyl, galactosyl, and fucosyl residues (reviewed in Fry, 1989a; Hayashi, 1989). Xyloglucans can form tight hydrogen bonds with the cellulose microfibrils (Valent and Albersheim, 1974; Hayashi et al., 1987, 1994a, 1994b; Hayashi, 1989) and therefore may form tethers between adjacent microfibrils, which are coiled around the cell (Fry, 1989b; Hayashi, 1989; McCann et al., 1990; Passioura and Fry, 1992). Modification of these xyloglucan cross-links may control the strength and extensibility of the cell wall. Recently, xyloglucan endotransglycosylase enzymes (XETs) (also called endoxyloglucan transferases [EXTs]) that modify xyloglucan polymers by internal cleavage and linkage of the newly generated ends to other xyloglucan polymer ends have been identified and purified from plants (Smith and Fry, 1991; Farkas et al., 1992; Fry et al., 1992; Nishitani and Tominaga, 1992; Fanutti et al., 1993). The ability of XETs to modify xyloglucan polymers has led to proposals that these enzymes function to incorporate newly synthesized

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xyloglucan during cell growth (Edelmann and Fry, 1992; Nishitani and Tominaga, 1992; Talbott and Ray, 1992), rearrange polymer tethers during cell wall loosening, leading to cell expansion (Fry, 1989b; Smith and Fry, 1991; Fry et al., 1992), modify primary cell walls (McCann et al., 1992), and partially lyse the cell wall, for example, at abscission zones, in developing xylem and phloem cells (Fry et al., 1992) or during fruit ripening (Redgwell and Fry, 1993). Thus, XETs may regulate cellular form and function through modification of the structure and properties of the cell wall.

The discovery of the touch (*TCH*) genes of Arabidopsis, which are strongly and rapidly induced in expression following a variety of environmental stimuli (Braam and Davis, 1990), introduces the possibility that the *TCH* gene products may have direct roles in eliciting environmentally induced developmental changes. *TCH1* encodes calmodulin (CaM; Braam and Davis, 1990), and *TCH2* and *TCH3* encode CaM-related proteins (Braam and Davis, 1990; Sistrunk et al., 1994; K.A. Johnson and J. Braam, unpublished results); their protein identities are more consistent with possible roles in signal transduction.

Here, we identify Arabidopsis TCH4 as an XET. TCH4 shows significant sequence identity to known XETs, and the TCH4 protein produced in *Escherichia coli* has XET activity. We show that expression of *TCH4* is regulated by the hormones auxin and brassinosteroids and by touch, darkness, and temperature shocks. During development, *TCH4* expression was localized in tissues undergoing expansion or cell wall modification. Hence, the plasticity of plant development may be controlled in part by cell wall changes. Regulation of genes encoding enzymes that alter cell wall properties may underlie the rapid adaptation of plants to environmental conditions.

RESULTS

TCH4 Is an XET

Figure 1 shows the complete transcription unit of the Arabidopsis *TCH4* gene. Using a partial (550-bp) cDNA for *TCH4* (Braam and Davis, 1990) as a probe, we isolated several genomic DNA and cDNA clones corresponding to the *TCH4* gene. The sequences of the longest cDNA of 1.071 kb, which contained a poly(A) tail, and the corresponding genomic region were determined. Comparing the cDNA and genomic sequences indicates that the *TCH4* gene contains a single intron of 84 bp within the coding region (Figure 1); the intron/exon boundaries follow the GT/AG rule (Mount, 1982). We identified the start sites of transcription using primer extension (data not shown) and found that *TCH4* mRNAs start with either of two A residues 49 and 50 bases upstream from the proposed translation initiation site (first and second residues shown in Figure 1). The full-length *TCH4* mRNA would therefore be expected to be ~1.1 kb long, consistent with the length of the *TCH4* mRNA detected on RNA gel blots (data not shown). The sequence TATATATA is found 30 bp upstream of the most 5′ transcription start site (data not shown); this sequence fits the consensus for a TATA box promoter element (Serfling et al., 1985). The first ATG codon is present at the start of the longest open reading frame encoding a 284–amino acid protein, and the flanking nucleotides are often found in plant initiator codons (Lutcke et al., 1987); therefore, this codon is probably used to initiate translation.

The deduced amino acid sequence of TCH4 is strikingly similar to a recently identified family of XET proteins. As shown in Figure 2, the putative TCH4 protein is 41.6, 52.2, and 64.8% identical in amino acid sequence to nasturtium, azuki bean, and tomato XETs, respectively; these enzymes have been shown directly to have xyloglucan endotransglycosylase activity (de Silva et al., 1993, 1994; Okazawa et al., 1993). Other *XET*-related genes are present in Arabidopsis, indicating that *TCH4* is a member of a multigene family. The putative protein products of Arabidopsis *EXT*, a gene isolated based on sequence similarity with azuki bean *XET* (Okazawa et al., 1993), and *Meri-5*, isolated as a gene expressed in meristems (Medford et al., 1991), are 48.6 and 57.4% identical, respectively, to TCH4. *XET*-related genes from other plants include soybean *BRU1*, which was isolated as a gene strongly upregulated in

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   1 AACCACAAACCAATCTAACTCAATACAAGAATATCTACAATCTCTAGAAAATGGCGATCA
                                                   M  A  I  T
  61 CTTACTTGCTTCCTCTGTTTCTTTCTCTTATCATCACCTCCTCTGTTTCAGCTAATTTCC
       Y  L  L  P  L  F  L  S  L  I  I  T  S  S  V  S  A  N  F  Q
 121 AAAGAGACGTTGAGATCACTTGGGGTGATGGTCGTGGACAGATCAAGAACAATGGAGAGC
       R  D  V  E  I  T  W  G  D  G  R  G  Q  I  K  N  N  G  E  L
 181 TTCTCACTTTATCTCTAGATAAATCCTCTGGTTCTGGATTCCAATCCAAAAACGAGTACT
       L  T  L  S  L  D  K  S  S  G  S  G  F  Q  S  K  N  E  Y  L
 241 TGTTTGGTAAAGTCTCCATGCAAATGAAGCTTGTCCCTGGAAACTCCGCAGGAACAGTCA
       F  G  K  V  S  M  Q  M  K  L  V  P  G  N  S  A  G  T  V  T
 301 CAACACTTTACgtaagttcaacaaaacaatctctgttttcgtttttgcatattataaagt
       T  L  Y
 361 tcttgtatctgaaatggtttttttgtatatgaacagTTGAAATCACCTGGAACAACATGGG
                                           L  K  S  P  G  T  T  W  D
 421 ACGAGATAGATTTCGAGTTTTTAGGGAATTCAAGTGGAGAACCTTACACACTTCACACAA
       E  I  D  F  E  F  L  G  N  S  S  G  E  P  Y  T  L  H  T  N
 481 ATGTCTACACACAAGGCAAAGGAGACAAAGAACAACAATTCAAACTCTGGTTTGATCCAA
       V  Y  T  Q  G  K  G  D  K  E  Q  Q  F  K  L  W  F  D  P  T
 541 CAGCTAATTTCCACACTTACACTATTCTCTGGAACCCACAAAGAATCATTTTCACCGTCG
       A  N  F  H  T  Y  T  I  L  W  N  P  Q  R  I  I  F  T  V  D
 601 ATGGAACTCCGATCAGAGAATTCAAGAACATGGAGTCTCTAGGCACTCTGTTTCCCAAGA
       G  T  P  I  R  E  F  K  N  M  E  S  L  G  T  L  F  P  K  N
 661 ACAAACCAATGAGAATGTACTCGAGTCTTTGGAACGCTGATGATTGGGCAACGAGAGGTG
       K  P  M  R  M  Y  S  S  L  W  N  A  D  D  W  A  T  R  G  G
 721 GTTTGGTCAAAACCGATTGGTCTAAAGCTCCTTTCACTGCTTCTTACCGTGGCTTTCAAC
       L  V  K  T  D  W  S  K  A  P  F  T  A  S  Y  R  G  F  Q  Q
 781 AAGAAGCTTGTGTTTGGTCAAACGGCAAGTCTTCTTGTCCTAATGCCTCGAAACAGGGGA
       E  A  C  V  W  S  N  G  K  S  S  C  P  N  A  S  K  Q  G  T
 841 CTACTACTGGCTCGTGGTTGTCACAAGAGCTTGACTCAACAGCTCAACAAAGGATGAGAT
       T  T  G  S  W  L  S  Q  E  L  D  S  T  A  Q  Q  R  M  R  W
 901 GGGTGCAGAGGAACTACATGATCTATAATTATTGTACGGATGCGAAGAGGTTCCCTCAAG
       V  Q  R  N  Y  M  I  Y  N  Y  C  T  D  A  K  R  F  P  Q  G
 961 GTCTTCCTAAAGAGTGCTTAGCTGCATAGAGAGAGTAAAGAGTTGAGAGAGGAACAAGAT
       L  P  K  E  C  L  A  A
1021 TTTATTTTTCTTTGTGGTTATAAAATTCTATTCATTTTATTGTAGATCACGTGAATTTTA
1081 TTGATTTGTTTTGTAGTATACTCTATAGTTCGTTAAAGTTATAATATTCTCTTTGTTACA
1141 A
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Figure 1. The *TCH4* Nucleic Acid Sequence and Deduced Amino Acid Sequence.

The sequence starts at the first transcription start site and ends with the last nucleotide before the poly(A) addition site. The predicted amino acid sequence is provided underneath the corresponding nucleic acid sequences. The predicted signal sequence cleavage site is after alanine (bold A) at position 21. The intron is indicated in lowercase letters. The GenBank accession number is U27609.

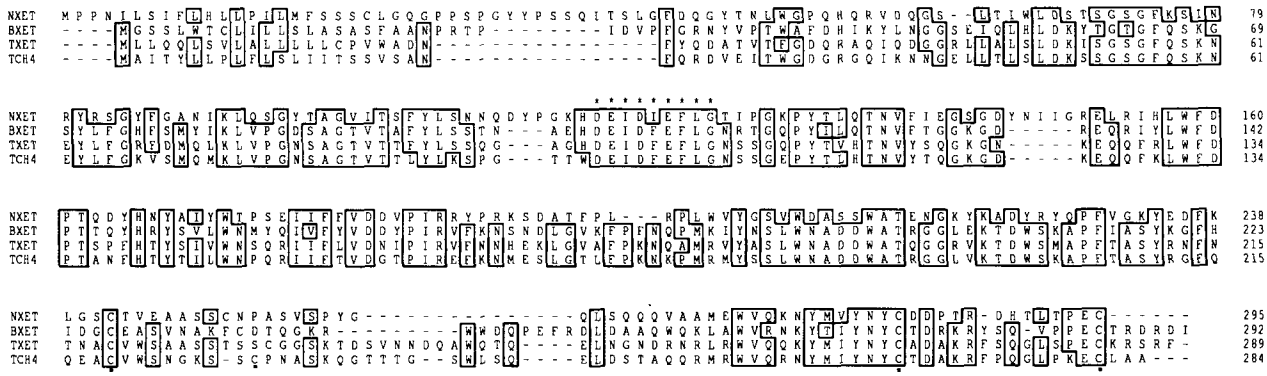

Figure 2. TCH4 Is Related to XETs.

Sequence alignment of XETs of nasturtium (NXET; de Silva et al., 1993), azuki bean (BXET; Okazawa et al., 1993), and tomato (TXET; de Silva et al., 1994) with TCH4. Amino acids shared by at least three proteins are boxed. Asterisks indicate a domain predicted to be important for catalytic activity. Conserved cysteines (C) are marked below the TCH4 sequence by black squares. Dashes indicate gaps to maximize alignment.

response to brassinosteroids (Zurek and Clouse, 1994), maize *wusl1005*, a flood-inducible gene (Peschke and Sachs, 1994), and *EXT*-related genes from soybean, tomato, and wheat (Okazawa et al., 1993); the proteins encoded by these genes are 48 to 64% identical in amino acid sequence to TCH4.

Consistent with the overall amino acid similarities between TCH4 and XETs, TCH4 harbors a potential signal sequence at the N terminus. This region lacks charged amino acids and is rich in hydrophobic residues; it therefore may function to target the protein across the endoplasmic reticulum membrane and, thus, ultimately to the cell wall. Cleavage of the potential signal sequence would most likely occur after alanine-21 residues from the N terminus (Figure 1), according to the predictions of von Heijne et al. (1986). TCH4 and the XET enzyme sequences are also similar to bacterial glucanases, especially the sequence DEIDF/IEFLG (asterisks in Figure 2). The first E of the DEIDIEFLG sequence in *Bacillus* β-glucanases has been proposed to be within the active site (Borriss et al., 1990) and therefore may be a critical amino acid for the cleavage of (1-4)-β-glycosyl linkages (de Silva et al., 1993; Okazawa et al., 1993). The N residue following this region in TCH4 and two of the other XET proteins may be glycosylated because this residue falls within the N-X-S/T consensus for N-linked glycosylation. The deduced TCH4 protein sequence also contains four C residues in the C-terminal portion of the protein that could form disulfide bridges; these four cysteines are found in all known XETs, although the position of the second C residue varies slightly (Figure 2).

To test the *TCH4*-encoded protein for XET activity, we produced a recombinant TCH4 protein in *E. coli*, denatured it with urea to solubilize inclusion bodies, and purified the recombinant protein via a C-terminal histidine tag using a nickel affinity column. The purified protein was dialyzed to remove the urea and allow renaturation of the protein. We assayed the ability of the recombinant TCH4 protein to catalyze the transfer of xyloglucan polymer segments to radioactively labeled xyloglucan-derived oligosaccharides to form labeled products that could be bound to cellulose filters (Fry et al., 1992). As shown in Figure 3, the rate of xyloglucan endotransglycosylation was nearly constant over the course of the experiment, with a 40-becquerel (Bq) product formed per hour per kilobecquerels (kBq) of substrate. The activity is relatively stable, persisting for more than 11 hr, despite the low protein concentration. Thus, TCH4 is capable of modifying a major component of the plant cell wall.

Expression of the *TCH4*-Encoded XET Is Regulated by Expansion-Promoting Hormones

One proposed function of endotransglycosylation of xyloglucan chains in the cell wall is to allow displacement of cellulose microfibrils under the force of turgor, resulting in cellular expansion. Plant growth regulators, such as auxin, brassinosteroids, and gibberellin, can enhance expansion or elongation of plant

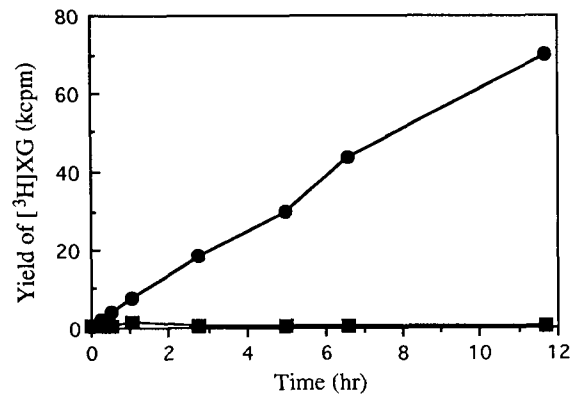

Figure 3. TCH4 Has XET Activity.

The TCH4 protein (105 μg/mL) was incubated with ^{3}H-labeled nonasaccharide in the presence (circles) or absence (squares) of nonradioactive xyloglucan (XG). Each point is the mean of duplicate assays. kcpm, kilo counts per minute.

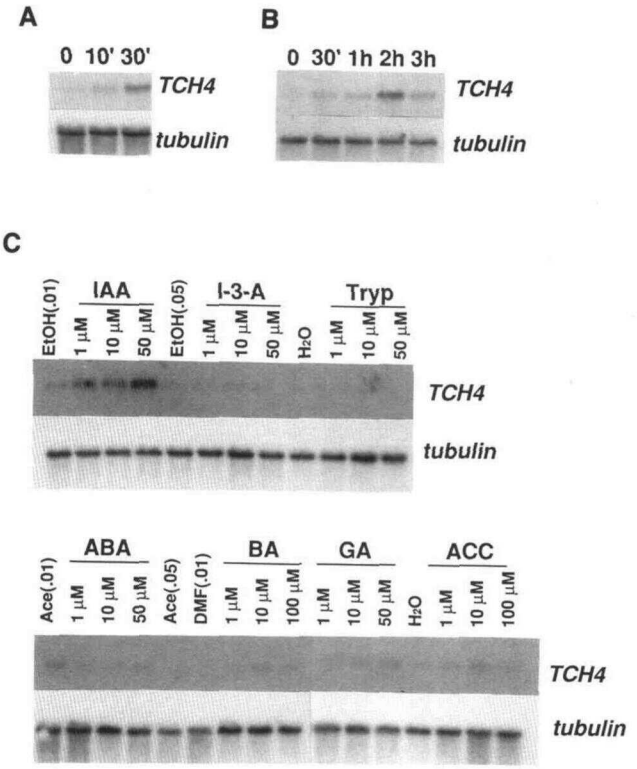

Figure 4. Upregulation of *TCH4* Expression by Auxin and Brassinosteroids.

(A) and **(B)** Plants were subjected to no treatment (lanes marked 0) or to 1 μM IAA **(A)** or BR **(B)** treatment for the indicated times. The prime indicates minute; h, hour.

(C) Indicated compounds were applied to plants as described in Methods. Plants were treated for 30 min with concentrations of compounds as indicated. As controls, solvents were added at the same volumes as for the experiments: 0.01% ethanol, EtOH (.01); 0.05% ethanol, EtOH (.05); water, H_2O; 0.01% acetone, Ace (.01); 0.05% acetone, Ace (.05); and 0.01% dimethyl formamide, DMF (.01). RNA was isolated, size fractionated on gels, blotted to filters, and hybridized with the probes indicated at right. I-3-A, indole-3-aldehyde; Tryp, tryptophan; ABA, abscisic acid; BA, benzyladenine; GA, GA_3; ACC, 1-aminocyclopropane-1-carboxylic acid.

tissue. Under some conditions, XET activity can be correlated with expansion regulated by auxin (Potter and Fry, 1994) and gibberellin (Potter and Fry, 1993). *BRU1*, a soybean gene that encodes an XET-related enzyme, shows strong post-transcriptional upregulation in plants treated with low concentrations of brassinosteroids (Zurek and Clouse, 1994). To test a possible regulatory link to plant growth hormones, we examined whether expression of *TCH4* is affected by various growth regulators. As shown in Figure 4A, the natural auxin indole-3-acetic acid (IAA) at 1 μm resulted in a significant increase in *TCH4* expression levels. This induction was rapid in that *TCH4* mRNA accumulated within 10 min (Figure 4A). Exposure of plants to 10 and 50 μM IAA resulted in similar or higher levels of *TCH4* mRNA accumulation, respectively (Figure 4C). At 1 μM, 24-epibrassinolide (BR), a highly active brassinosteroid (Mandava, 1988), also resulted in *TCH4* upregulation; however, the kinetics of induction by BR were slower than those of the auxin induction, with mRNA accumulation peaking at 2 hr after treatment (Figure 4B). The inactive auxin-related compounds indole-3-aldehyde and tryptophan failed to result in increased *TCH4* mRNA accumulation at 1, 10, or 50 μM (Figure 4C). Abscisic acid, cytokinin (benzyladenine), gibberellin A_3 (GA_3), and 1-aminocyclopropane-1-carboxylic acid (which is constitutively converted to ethylene; Rothenberg and Ecker, 1993) had no detectable effect on *TCH4* expression (Figure 4C). Thus, *TCH4* expression can be induced specifically by auxin and brassinosteroid and may be regulated during development by endogenous hormone levels. Furthermore, the *TCH4*-encoded XET may mediate hormone-regulated morphogenetic processes.

A 5′ *TCH4* DNA Fragment Is Sufficient To Confer Touch, Darkness, Temperature Shock, Auxin, and Brassinosteroid Inducibility

Arabidopsis *TCH4* gene expression has been shown to be regulated by various environmental stimuli, including touch, darkness, heat shock, and cold shock (Braam and Davis, 1990; Braam, 1992; D.H. Polisensky and J. Braam, unpublished data), in addition to being regulated by auxin and brassinosteroids, as discussed above. To begin to identify the sequences responsible for this regulation, we fused ~1 kb of *TCH4*, including 958 bp upstream from the proposed first transcription start site, and 48 bp of the 5′ untranslated region to the *E. coli* β-glucuronidase (*GUS*) reporter gene. This fusion gene was introduced into Arabidopsis cells, and transgenic plants were generated. DNA gel blot analysis was used to identify transgenic plants that resulted from independent transformation events (data not shown). Control reporter transgenes lacking *TCH4* sequences failed to show upregulation in response to stimulation (data not shown).

We tested whether this 5′ fragment of the *TCH4* locus is sufficient to confer inducibility of expression to the *GUS* reporter gene. One time point after stimulation was chosen for these analyses. *TCH4* showed high levels of expression 15 min after stimulation by touch or darkness and 30 min after heat and cold shock treatments (data not shown). Three independent transgenic lines (designated 1, 2, and 3 in Figure 5) were stimulated by touch or darkness (Figure 5A), heat shock (Figure 5B), or cold shock (Figure 5C). Induction of expression of the reporter gene (*GUS*) is comparable to the regulation of the endogenous *TCH4* gene. Inducibility of expression by auxin and brassinosteroid was also conferred by this DNA region because, as shown in Figures 5D and 5E, *TCH4–GUS* mRNA accumulated in unison with *TCH4* mRNA following exposure of plants to 1 μM IAA or 1 μM BR. Thus, the 1-kb region of *TCH4* also confers auxin and brassinosteroid inducibility and

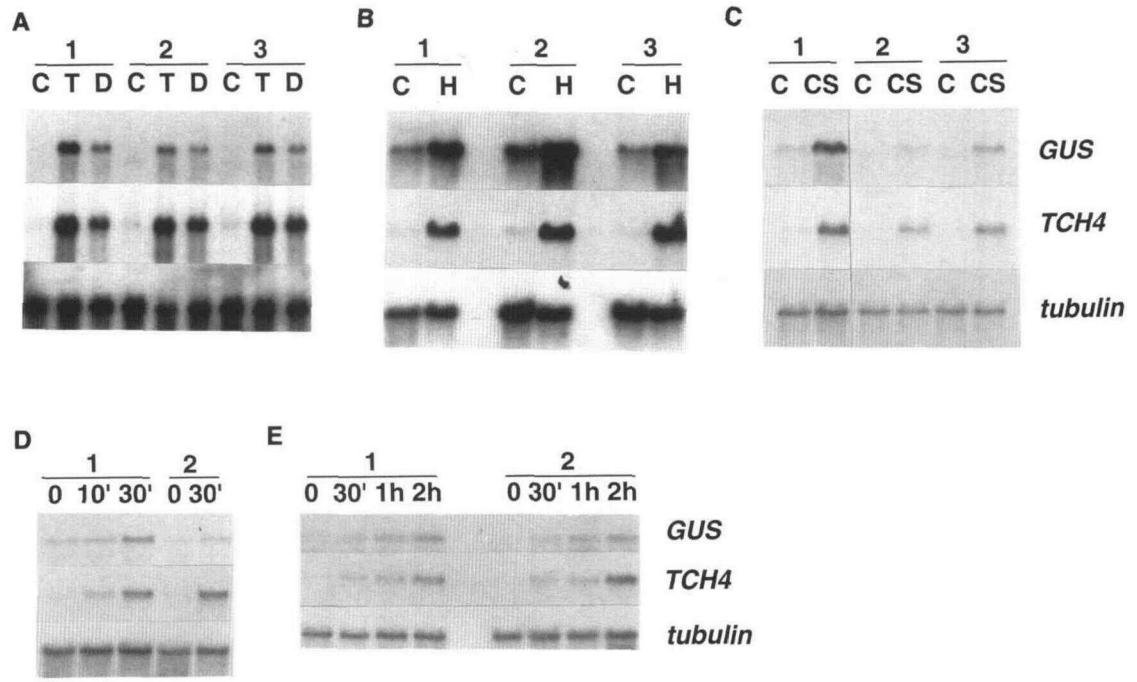

Figure 5. One Kilobase of the *TCH4* Gene Is Sufficient for Induction of Expression by Touch, Darkness, Heat Shock, Cold Shock, Auxin, and Brassinosteroids.

(A) to **(C)** RNA isolated from plants harvested 15 min after no stimulus (C), touch (T), or darkness (D); 30 min after no stimulus (C) or heat shock (H); and 30 min after no treatment (C) or cold shock (CS), respectively.

(D) RNA isolated from plants harvested 30 min after the addition of solvent (ethanol) 0 or at the indicated times following 1 μM IAA treatment.

(E) RNA isolated from plants harvested 30 min after the addition of solvent (ethanol) 0 or at the indicated times following treatment with 1 μM BR *treatment*.

RNA was size fractionated on gels, blotted to filters, and hybridized with probes at right. The numbers at the top indicate independent transgenic lines. The prime indicates minute; h, hour.

is sufficient for the proper kinetics of hormone-induced *TCH4* transcript accumulation.

TCH4 Is Expressed in Young Expanding Leaves, Trichomes, Lateral Root Primordia, Vascular Tissue, Abscission Zones, and Elongating Hypocotyls

XETs have been proposed to function in the process of cell expansion (Fry, 1989b; Smith and Fry, 1991; Fry et al., 1992) and cell wall biosynthesis (Edelmann and Fry, 1992; Talbott and Ray, 1992) and modification (Fry et al., 1992; McCann et al., 1992; Redgwell and Fry, 1993), and increased levels of XET activity have been correlated with regions of growth (Fry et al., 1992; Hetherington and Fry, 1993; Potter and Fry, 1993, 1994; Pritchard et al., 1993). To determine whether the 1-kb *TCH4* DNA fragment confers organ- or tissue-specific regulation to the *GUS* reporter gene, we examined the localization of GUS activity in the transgenic plants. The patterns of expression, examples of which are shown in Figure 6A through 6G, were indistinguishable for independent transgenic plants. Staining for GUS activity in control plants harboring the *GUS* gene driven by either the relatively constitutive 35S cauliflower mosaic virus promoter or no promoter provides evidence that the staining patterns in plants transformed with *TCH4–GUS* were due to the *TCH4* sequences and are not a property of GUS itself or the availability of substrate (data not shown).

Young expanding leaves showed high levels of *TCH4*-driven GUS activity, whereas activity was greatly reduced in leaves that are fully expanded (Figure 6A). The single-cell hair-like structures, called trichomes, stained strongly for GUS activity (Figure 6B). In roots, expression of the *TCH4–GUS* gene was most prominent in emerging lateral root primordia, where pericycle cells actively divide and expand (Figures 6C and 6D). The primary root tip was also often stained, indicating GUS activity (data not shown). The vascular tissues of roots (Figure 6C) and shoots (data not shown) also showed significant *TCH4*-driven GUS activity. In flowers, the pistils consistently showed GUS activity (Figure 6E), whereas GUS activity in the stigma was found only infrequently (data not shown). GUS activity was highest in seed pods (siliques) undergoing elongation and decreased with advanced development; however, the abscission zone at the base of siliques consistently expressed the *TCH4–GUS* gene (Figure 6F). *Meri-5*, an Arabidopsis gene

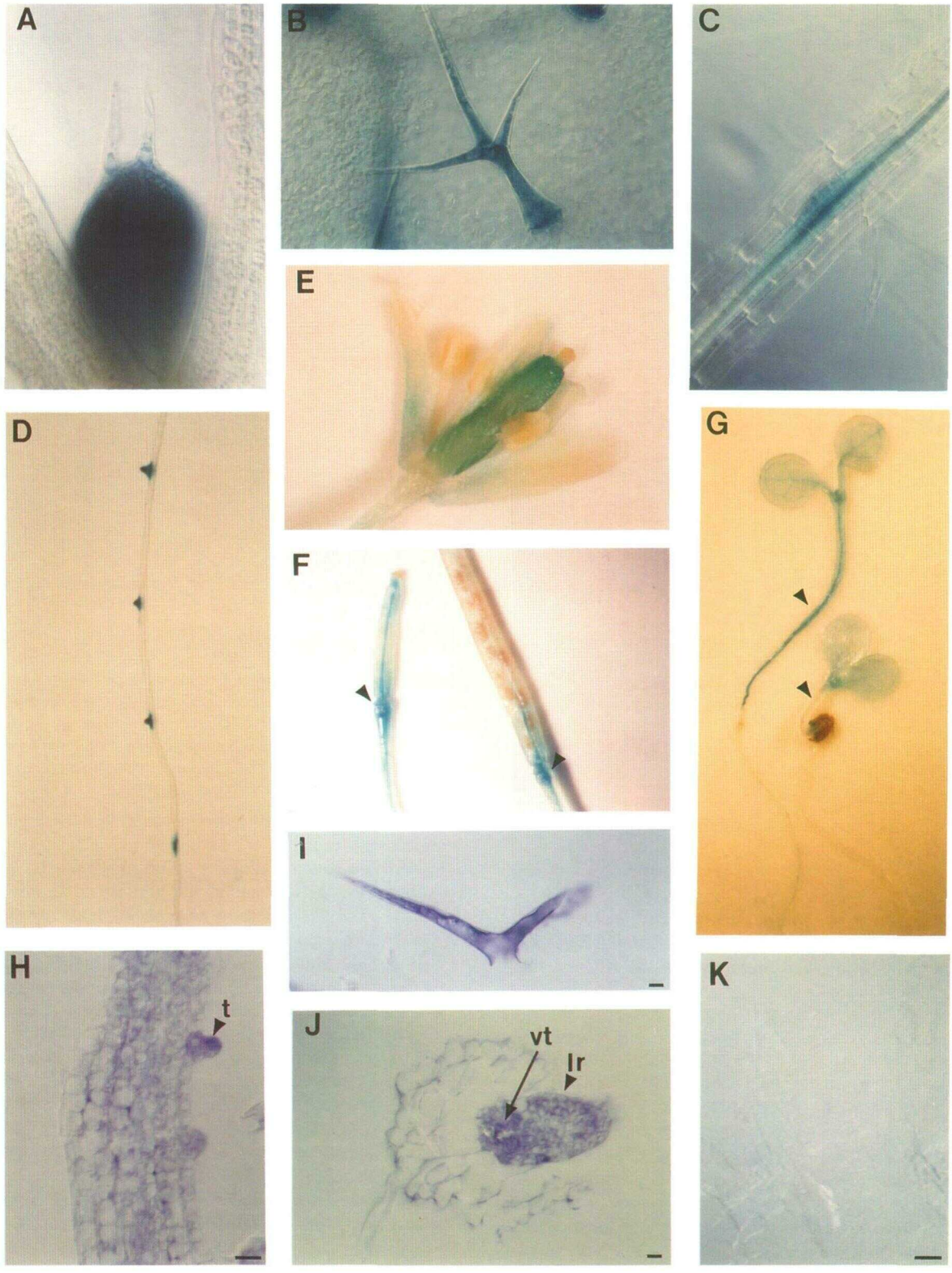

Figure 6. Developmental Expression of the *TCH4–GUS* Reporter Gene and Localization of TCH4 Protein.

closely related to *TCH4* in sequence, is most likely expressed with a different developmental pattern than *TCH4*; Medford et al. (1991) found that the strongest expression of *Meri-5–GUS* expression is at branching points of the root and shoot.

When Arabidopsis seedlings were grown under low light (~200 footcandles), the hypocotyls underwent extensive elongation. These elongated hypocotyls stained strongly, indicating high levels of GUS activity in comparison with less elongated hypocotyls of plants grown under 500 footcandles (Figure 6G).

We used immunodetection of the TCH4 protein itself to determine whether *TCH4*-driven GUS activity patterns reflected native *TCH4* expression. The antibody used to detect TCH4 was raised against the C-terminal region of TCH4 (see Methods). This region is more divergent in amino acid sequence from other known Arabidopsis XET-like proteins (W. Xu, P. Campbell, and J. Braam, unpublished results). This antibody recognized a single band on protein gel blots (D.M. Antosiewicz, M.M. Purugganan, and J. Braam, unpublished results). Reaction with preimmune serum gave no detectable staining (Figure 6K). We found that the pattern of TCH4 protein accumulation closely mimicked *TCH4–GUS* expression patterns. High levels of TCH4 protein were detected in young leaf primordia (Figure 6H), trichomes (Figures 6H and 6I), emerging lateral root primordia (Figure 6J), and the root vascular tissue (Figure 6J). There was no significant immunoreaction at branching points, indicating that the antibody would likely fail to interact with Meri-5. Because Meri-5 and TCH4 are among the most similar in sequence of the known XET-like proteins of Arabidopsis (W. Xu, P. Campbell, and J. Braam, unpublished results), it is probable that the antibody used for these analyses is specific for the TCH4 protein.

DISCUSSION

TCH4 Encodes an XET and Is a Member of a Multigene Family

Based on sequence similarity and in vitro activity of a recombinant TCH4 protein, we identified TCH4 as an XET. These results indicate that the rapid and dramatic regulation of *TCH4* gene expression may lead directly to modification of the structure and properties of cell walls. Cell wall changes are most likely central to developmental alterations that result in adaptation to environmental conditions.

TCH4 is 42 to 65% identical on the amino acid level to other proteins shown to have XET activity. Sequences thought to be critical for activity are highly conserved among the XETs and are present in the TCH4 protein. The specific activity of the bacterially produced recombinant TCH4 protein (6.8 Bq/kBq/hr/μg) was substantially lower than that in extracts of several vegetative plant tissues (e.g., 35 Bq/kBq/hr/μg in crude extracts of pea stems; Fry et al., 1992). This low specific activity of the purified recombinant TCH4 protein may have resulted from the following: (1) the presence of additional amino acids at the protein termini encoded by the expression vector; (2) the absence of possible plant cell–specific post-translational modifications, such as glycosylation; (3) the incomplete renaturation during urea removal; (4) the use of assay conditions optimized for a different isoenzyme of XET; and (5) the possibility that TCH4 protein predominantly catalyses the transglycosylation of substrates different from those tested.

The N terminus of TCH4 includes a putative signal sequence for transmembrane transport that would most likely target TCH4 to the cell wall. Indeed, light microscopic immunolocalization showed that the highest TCH4 accumulation is at cellular juxtapositions consistent with an extracellular location (D.M. Antosiewicz, M.M. Purugganan, and J. Braam, unpublished results).

TCH4 also shows significant similarity with other Arabidopsis genes, namely, *Meri-5* and *EXT*, as well as with a number of cDNAs that have been partially sequenced by the Arabidopsis genome project (Höfte et al., 1993; Newman et al., 1994). An *XET*-related gene family also exists in tomato (de Silva et al., 1994). The significance of *XET*-related gene families is not yet understood. The different gene products may have related but distinct specificities for substrates or sites of endopolysaccharide cleavage and transglycosylation. Alternatively, the proteins may function in distinct regions of the cell wall or subset of cells, tissues, or organs or in response to particular environmental or developmental cues. For example, the developmental patterns of *TCH4–GUS* expression and TCH4 protein accumulation are not identical to the expression pattern of a *Meri-5–GUS* reporter gene (Medford et al., 1991); thus, *TCH4* and *Meri-5* probably are regulated differentially during development.

Figure 6. (continued).

(A) to **(G)** Transgenic Arabidopsis plants harboring *TCH4–GUS* genes stained for GUS activity. In **(A)**, the first pair of true leaves flanked by fully expanded seed leaves (cotyledons) is shown. **(B)** shows the leaf trichome. In **(C)**, the region of the root with initiation of the lateral root primordium is shown. **(D)** shows the primary root with multiple lateral root primordia. **(E)** shows the flower, and in **(F)** are the siliques (seed pods), with arrowheads indicating abscission zones. In **(G)**, the seedling at left was grown under 200 footcandles of light; the seedling at right was grown with 500 footcandles of light. Arrowheads indicate hypocotyl regions.

(H) to **(J)** Immunolocalization of the TCH4 protein in nontransgenic plants. **(H)** shows a longitudinal section of a leaf primordium. t, developing trichome. **(I)** shows a longitudinal section of a leaf trichome. In **(J)** is a root cross-section. vt, vascular tissue; lr, lateral root primordium.

(K) Longitudinal section of an epicotyl with leaf primordia reacted with preimmune serum.

In **(H)** to **(J)**, bars = 100 μm; in **(K)**, bar = 10 μm.

TCH4 May Modify Xyloglucan Polymers in Response to Auxin, Brassinosteroids, and Environmental Stimuli

Modification of xyloglucan polymer length and arrangement likely occurs to regulate formation of such specialized cell walls and properties as cell wall extensibility and strength. Talbott and Ray (1992) have described the dynamic nature of xyloglucan chain length in isolated pea internode segments. Consistent with the findings of others (Labavitch and Ray, 1974; Nishitani and Masuda, 1983; Inouhe et al., 1984; Lorences and Zarra, 1987; Wakabayashi et al., 1991), they found a significant decrease in the molecular mass peak of xyloglucan chains in response to low levels of IAA. In addition, in the absence of added IAA, internode segments experienced increases in xyloglucan chain lengths, perhaps in response to wounding or sudden turgor changes (Talbott and Ray, 1992). It is not known how these changes in xyloglucan are generated; however, XET action is a possibility. Products of XET-encoding genes, such as *TCH4*, that are upregulated in expression by both auxin and mechanical perturbations may therefore be responsible for the resulting cell wall changes. Indeed, *TCH4* upregulation occurs very quickly, with significant mRNA accumulation occurring within 10 to 15 min after stimulation (Figures 4 and 5; Braam and Davis, 1990); these kinetics are consistent with the time course of the wall modifications (Taiz, 1984).

Brassinosteroids have been shown to increase the relaxation properties of *Brassica* cell walls (Wang et al., 1993) and the plastic extensibility of soybean epicotyl segments (Zurek et al., 1994). These changes most likely require the action of polysaccharide-modifying enzymes, and XET-like proteins encoded by BR-induced genes, such as *BRU1* (Zurek and Clouse, 1994) and *TCH4*, are attractive candidates for catalyzing these cell wall modifications.

Possible Physiological Functions of TCH4

Enzymes with XET activity may control cell wall loosening and cell expansion (Fry, 1989b; Smith and Fry, 1991; Fry et al., 1992), partial cell wall lysis (Fry et al., 1992; Redgwell and Fry, 1993), and cell wall formation (Edelmann and Fry, 1992; Talbott and Ray, 1992) and strengthening (through the generation of longer xyloglucan chains; Talbott and Ray, 1992). XETs may also play a role in controlling the availability of oligosaccharide signaling molecules. Specific xyloglucan fragments have been shown to have potent anti-auxin activity, and XETs could be involved in the generation and loss of these molecules through transglycosylation to xyloglucan polymers (Baydoun and Fry, 1989; Aldington and Fry, 1993).

The regulation of expression of *TCH4* by the growth-promoting hormones auxin and brassinosteroids is consistent with the possibility that the *TCH4*-encoded XET may modify xyloglucan chains in cell walls to control hormone-induced cell expansion. In many plant species, auxin-induced elongation has been reported to begin 10 to 15 min after auxin treatment, with a maximum rate reached within 30 to 45 min (Taiz, 1984). In contrast, enhanced elongation by brassinosteroids in soybean epicotyls can first be detected 45 min after treatment and does not reach maximum levels for several hours (Zurek et al., 1994). Interestingly, *TCH4* expression is more rapidly upregulated by IAA, with a maximum response occurring after 30 min; however, the *TCH4* response to BR is somewhat delayed, with maximum levels of mRNA accumulating 2 hr after treatment. Thus, the kinetics of *TCH4* mRNA accumulation by these hormones mirror their expansion-inducing kinetics; this correlation suggests that TCH4 may participate in eliciting the hormone-regulated expansion.

XET activity levels can be correlated with growth regulated by auxin (Potter and Fry, 1994) or gibberellin (Potter and Fry, 1993). However, levels of XET activity are not always correlated with growth (Fry et al., 1992; Hetherington and Fry, 1993). Excised pea stem segments, whose elongation is promoted by auxin at micromolar concentrations, do not exhibit any auxin-induced increase in extractable XET activity. Furthermore, protein extracts rich in XET activity are incapable of eliciting extension in isolated cucumber hypocotyl segments (McQueen-Mason et al., 1993). Other proteins, called expansins, have been identified recently that are sufficient to cause extension in this in vitro extension assay (McQueen-Mason et al., 1992), yet these proteins lack XET activity (McQueen-Mason et al., 1993). Thus, other proteins unrelated to XETs likely play critical roles in plant cell expansion and growth. The potential roles of XETs in cell expansion remain to be proven.

The in situ localization of *TCH4* expression leads to insight into the possible developmental functions of the *TCH4*-encoded XET. Again, there is a correlation of *TCH4* with expansion because *TCH4* expression appears to be prominent in regions where cellular expansion is likely occurring. That is, *TCH4–GUS* genes were strongly expressed (Figure 6A) and TCH4 protein accumulated (Figure 6H) in young expanding leaves, trichomes (Figures 6B and 6I), and lateral root primordia (Figures 6C, 6D, and 6J). Under low-light conditions, *TCH4–GUS* was expressed throughout the elongated hypocotyl (Figure 6G). Cells likely undergoing cell wall modification, such as those in the developing vascular tissue and abscission zones, also expressed *TCH4–GUS* fusion genes and accumulated TCH4 protein (Figures 6C, 6F, and 6J). Changes in vascular cell walls include strengthening and partial lysis to generate perforations in the walls of developing xylem and phloem. Expression of *TCH4* at these sites suggests that the *TCH4*-encoded XET may function in these processes. Endogenous auxin levels are believed to influence both cell expansion and vascular tissue development; therefore, the finding that exogenous auxin induced *TCH4* expression is also consistent with a role of the *TCH4*-encoded XET in cell expansion and vascular tissue development.

Paradoxically, in response to mechanical stimulation, *TCH4* expression is increased, yet the plants become less elongated than plants grown without stimulation (Braam and Davis, 1990). It is possible that the TCH4 protein promotes expansion under these conditions, but in mechanically stimulated plants, the expansion that occurs is limited to a radial direction.

Alternatively, TCH4 XET activity may be redirected toward strengthening cell walls and may play a role in the mechanically induced generation of stronger and/or more flexible plants (Biddington, 1986; Telewski and Jaffe, 1986a, 1986b; Jaffe and Forbes, 1993; Mitchell and Myers, 1995). Xyloglucan chains of the cell wall likely contribute rigidity and mechanical strength to plant tissue (Hayashi, 1989). Indeed, mutants of Arabidopsis that are deficient in fucose, a component of xyloglucans, show dramatically less wall strength than wild-type plants (Reiter et al., 1993). During development, specific regions of the plant, such as branching points, would likely experience mechanical strain, and as a result, the cell walls of these particular tissues may be reinforced. The *Meri-5*–driven *GUS* activity pattern (Medford et al., 1991), which may reflect endogenous *Meri-5* expression, includes prominent GUS activity at branch points throughout the plant. Sistrunk et al. (1994) described branching point expression of *TCH3–GUS* genes and accumulation of the CaM-related TCH3 protein. These results suggest that the *TCH3* and *Meri-5* gene products function at these locations to increase strength or flexibility to withstand mechanical strain generated by the weight of the branching structure. In addition, because *TCH3* (Braam and Davis, 1990; Sistrunk et al., 1994) and *Meri-5* (W. Xu and J. Braam, unpublished results) are touch inducible, expression of these genes during development may also be regulated by the endogenous mechanical strains generated during morphogenesis. Stem–petiole junctions have been shown to have increased cross-linking (Bradley et al., 1992) or accumulation (Tiré et al., 1994) of hydroxyproline-rich structural proteins; thus, plant cells may have multiple mechanisms of reinforcing cell walls. *TCH4–GUS* expression was not prominent at branch points, again suggesting that the expression of the *XET*-related gene family of Arabidopsis is differentially regulated during development.

Environmental, Hormonal, and Developmental Regulation of *TCH4* Expression through a 1-kb DNA Fragment

We showed that a 1-kb fragment of the *TCH4* genomic locus was sufficient to cause upregulation of reporter gene expression following the diverse environmental stimuli of touch, darkness, cold shock, and heat shock. In addition, auxin and brassinosteroid regulation was controlled by this DNA fragment. A question that remains is, How do such diverse stimuli cause a common molecular response? It is possible that distinct signal transduction pathways act to control *TCH4* expression following each of the different stimuli. Alternatively, the signal transduction pathways may converge at some point before *TCH4* regulation. Some evidence suggests that an increase in free cytoplasmic calcium ions may serve as a common second messenger in response to at least some of these diverse stimuli and signal an increase in *TCH4* gene expression (Braam, 1992).

The 1-kb DNA region was also sufficient to confer appropriate developmental-specific expression onto the *GUS* reporter gene. That is, a *TCH4–GUS* fusion gene showed an expression pattern very similar to the pattern of TCH4 protein accumulation in nontransgenic plants.

It will be interesting to dissect further this 1-kb DNA region to determine whether there is a common *cis* regulatory element that functions to confer inducibility to all the inducing stimuli. Although many auxin-regulated genes have been shown to be controlled by altering the rate of initiation of transcription (McClure et al., 1989), *BRU1* regulation by brassinosteroids is thought to occur post-transcriptionally (Zurek and Clouse, 1994). Thus, it is possible that *TCH4* is regulated both transcriptionally and post-transcriptionally, depending on the inducing stimulus. Sequences controlling any post-transcriptional regulation by BR would have to be located within the first 48 bases of the transcription unit, because the *TCH4–GUS* fusion genes possess the BR-sensitive regulation of expression. Identification of *trans*-acting factors will help elucidate the signal transduction pathways by which environmental stimulation leads to gene regulation. Finally, the use of mutants unable to respond to specific stimuli should shed light on the relationship between hormonal, developmental, and environmental regulation of *TCH4* expression.

Conclusion

This identification of TCH4 as an XET provides insight into the physiological relevance of the strong and rapid regulation of expression of the Arabidopsis *TCH* genes by environmental stimuli. The placement, mechanical properties, and regulation of expansion—in direction and magnitude—of the cell wall are fundamental in determining plant form. Thus, environmental regulation of the expression of genes such as *TCH4,* whose protein products most likely directly alter cell wall properties, may be a critical mechanism by which plant tissue properties are altered in response to changes in the environment.

METHODS

Plant Growth and Manipulations

Plants were maintained at 24°C in 65 to 75% humidity under constant light. For generation of transgenic plants, *Arabidopsis thaliana* ecotypes Nossen (No-O) or RLD plants were transformed as described by Valvekens et al. (1988). Independent transformants were determined by DNA gel blot hybridization. Analyses were performed on third generation plants homozygous for kanamycin resistance.

Touch, darkness, and heat shock treatments were conducted as described previously (Braam and Davis, 1990; Braam, 1992). Cold shock treatment was conducted using cultured cells generated as described (Braam, 1992). To elicit a sudden cold shock, flasks containing 10 mL of cells in media were gently swirled in ice water for 2 min to bring the temperature of the media rapidly to 0°C; the flasks were then swirled

in room temperature water for 2 min to rewarm the media. Subsequently, the flasks were placed on a rotary shaker at 120 rpm for 26 min. As controls, cells were treated exactly as described, except that the first 2-min incubation was conducted in a room temperature water bath.

Plants to be treated with indole-3-acetic acid (IAA) or 24-epibrassinolide (BR) (a gift of S.D. Clouse, North Carolina State University, Raleigh, NC) were grown for ~12 days in 0.5 × Murashige and Skoog salts (Sigma), 1 × Gamborg's vitamins (Sigma), and 1% sucrose, pH 5.7, on a rotary shaker at 120 rpm with 24 hr of light at 24°C. Twelve to 16 hr before the experiment, the medium was replaced with 20 mL of fresh medium. Hormones and auxin-related compounds were added to the media without disturbing the flasks. Controls consisted of adding only solvents. IAA and BR samples were collected at the time points indicated in Figures 4 and 5; samples with added abscisic acid, benzyladenine, gibberellin A_3 (GA_3), 1-aminocyclopropane-1-carboxylic acid, tryptophan, and indole-3-aldehyde were harvested at 30 min. Collection involved quickly decanting the media and submerging the plants into liquid nitrogen. Samples were stored at −80°C prior to RNA purification.

Transgenic plants were stained for β-glucuronidase (GUS) activity essentially according to Gallagher (1992). Briefly, plants were fixed in 2% paraformaldehyde, 100 mM sodium phosphate, pH 7.0, and 1 mM EDTA on ice for 30 min. After the plants were washed in 100 mM sodium phosphate, pH 7.0, they were submerged and vacuum infiltrated in 2 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-gluc; gift from Molecular Probes, Eugene, OR) in 50 mM sodium phosphate, pH 7.0, and 0.5% Triton X-100 for 2 min and subsequently incubated at 37°C for several hours. After the reaction was stopped with water, the plants were bleached several times by washing with 70% ethanol.

For immunolocalization analysis of shoot material, plants were grown in soil. For growth of sterile roots, plants were grown in Petri plates with 0.5 × Murashige and Skoog salts, 1 × Gamborg's vitamins, and 1% sucrose, pH 5.7.

DNA and RNA Manipulations

A partial *TCH4* cDNA (~550 bp; Braam and Davis, 1990) was labeled with digoxigenin using the Boehringer Mannheim Genius kit and used to probe Arabidopsis genomic and cDNA libraries (Sambrook et al., 1989). Restriction mapping and subcloning were perfomed according to Sambrook et al. (1989). DNA sequence analysis was determined by the dideoxynucleotide chain termination method (Sanger et al., 1977) or was performed by the Nucleic Acids Core in the Department of Molecular and Human Genetics at Baylor College of Medicine (Houston, TX) using automated fluorescence DNA sequencing techniques (Smith et al., 1986). Both DNA strands were sequenced in their entirety. Sequence alignments and analyses were performed using software from Genetics Computer Group (version 8; Madison, WI; Devereux et al., 1984) and the BLAST network service from the National Center for Biotechnology Information (Altschul et al., 1990).

For primer extension, a synthetic oligonucleotide, 5′-GTGATCGCCATTTTCTAGAGATTGTAGATATTC-3′, complementary to the sequence beginning two bases from the 5′ end of the longest cDNA, was radiolabeled with T4 polynucleotide kinase (U.S. Biochemical), according to the manufacturer's specifications. Fifteen picomoles of primer was extended by Moloney murine leukemia virus reverse transcriptase (U.S. Biochemical) in the presence of total RNA from plants stimulated by touch. The oligonucleotide was also used for sequencing (Sequenase kit; U.S. Biochemical). Reaction products were subjected to electrophoresis through a 8% polyacrylamide–urea gel, and autoradiography was performed.

For RNA gel blots, total RNA was purified (Verwoerd et al., 1989), subjected to electrophoresis on formaldehyde gels, and transferred to filters, as described previously (Braam and Davis, 1990). Filters were probed with hexamer-labeled DNA fragments (Feinberg and Vogelstein, 1983). Probes were derived from a 1.8-kb BamHI-SacI fragment of *GUS* from the vector pBI221 (Clontech, Palo Alto, CA), a partial *TCH4* cDNA (Braam and Davis, 1990), and a BamHI-KpnI fragment of an Arabidopsis β-tubulin (Marks et al., 1987).

The *TCH4–GUS* transcriptional fusion gene was constructed by ligation of a 1-kb XbaI genomic *TCH4* fragment into the XbaI site of the *GUS* reporter vector pBI101.2 (Clontech; Jefferson et al., 1987). The recombinant plasmid was transformed into *Agrobacterium tumefaciens* LBA4404 via electroporation.

Production of Recombinant TCH4 Protein in Bacteria

The DNA region encoding the putative mature TCH4 polypeptide (lacking the putative signal sequence) was amplified by polymerase chain reaction (PCR) using the primers 5′-GCTGGATCCAATTTCCAAAGAG-3′ and 5′-GTCGAGCTCAATGCAGCTAAGC-3′. The PCR product was digested with BamHI and SacI (sites engineered at the 5′ and 3′ ends, respectively), and the product was ligated into pET21 (Novagen, Madison, WI) to generate a fusion protein with six histidine residues at the C terminus. The resulting plasmid, pT4Mat1, was transformed into DH5α. The target gene was expressed by infecting *Escherichia coli* with bacteriophage CE6 (Novagen). The cells were pelleted after 3 hr and frozen at −80°C. Cells were lysed with 100 μg/mL lysozyme and 0.1% Triton X-100 in buffer A (20% glycerol, 0.5 M NaCl, and 0.04 M Tris-HCl, pH 7.4). Soluble and insoluble proteins were separated by centrifugation at 15,000*g* for 20 min. The insoluble protein fraction, which contained most of the recombinant TCH4 protein, was incubated on ice for 1 hr in buffer A with 8 M urea. Solubilized proteins were isolated in the supernatant following centrifugation at 39,000*g* for 20 min and then loaded onto a nickel-charged column (Novagen). The column was washed with a reverse urea gradient (8 to 1 M) in buffer A to allow refolding of the denatured protein and then was washed with 0.02 M imidazole and 1 M urea in buffer A. TCH4 was eluted with 0.3 M imidazole and 1 M urea in buffer A. Fractions containing protein were pooled, diluted to 0.08 mg/mL in buffer A, and dialyzed against buffer A to remove the urea and imidazole. The protein was concentrated in a Centriprep-10 ultrafiltration device (Amicon, Beverly, MA). Protein concentration was estimated by the Micro-BCA kit (Pierce, Rockford, IL) using BSA as a standard.

Materials and Conditions for Xyloglucan Endotransglycosylase Assays

Xyloglucans were used as the donor substrate in xyloglucan endotransglycosylase (XET) assays. *Tamarindus* seed xyloglucan was a generous gift of J.S.G. Reid (University of Stirling, Stirling, UK). *Tropaeolum* seed xyloglucan was extracted and purified as described by McDougall and Fry (1990). The nonasaccharide XLLG (X stands for xylosyl-substituted glucose, L for galactosyl-xylosyl–substituted glucose, and G for unsubstituted glucose; for a full explanation of nomenclature, see Fry et al., 1993) was produced by digestion of *Tropaeolum* xyloglucan with cellulase and purified by gel permeation chromatography on a Bio-

Gel P-2 column (Bio-Rad); XLLG was then reduced with NaB^3H_4 to yield labeled ^{3}H-XLLGol (Gol stands for glucitol) with a specific activity of 37.4 MBq/mol. The oligosaccharides were repurified on Bio-Gel P-2.

Aliquots (8 μL of the solution to be tested containing 10% glycerol, 0.5 M NaCl, and 0.04 M Tris-HCl, pH 7.4, with or without the TCH4 protein at 740 μg/mL) were mixed with 48 μL of a substrate mixture to give the following final concentrations: 2.6 mg/mL ^{3}H-XLLGol (acceptor substrate), ~10.7 μM of which was ~22.4 kBq per assay; succinate buffer at 0.137 M (adjusted with Na^+ to pH 5.5) with or without *Tamarindus* xyloglucan (donor substrate) and with or without TCH4 protein (105 μg/mL). After incubation at 22°C for the times indicated in Figure 3, the reaction was stopped by adding 40 μL of 90% formic acid, and the solution was dried onto a 4 × 4–cm square of Whatman 3MM filter paper. The paper was washed in running tap water for 1 hr to remove the remaining ^{3}H-XLLGol, redried, and assayed for bound ^{3}H by scintillation counting in 2 mL of 0.5% 2,5-diphenyloxazole (BDH Laboratory Supplies, Poole, Dorset, UK) per 0.05% 1,4-di-2-(5-phenyloxazoyl) benzene (BDH Laboratory Supplies) in toluene (counting efficiency of ~40%).

Antibody Production and Immunohistochemistry

The entire protein-coding region of *TCH4* was amplified by PCR, using the primers 5′-GCGGGATCCATGGCGATCACT-3′ and 5′-GTCGAGCTCAATGCAGCTAAGC-3′. The PCR product was digested with EcoRI (an internal *TCH4* site) and SacI (a site engineered at the 3′ end) to generate a 370-bp fragment encoding the C-terminal region of TCH4, which was then ligated into pET21. The resulting plasmid, pMP2, was transformed into BL21(*pLys*)S cells (Novagen). The manufacturer's directions were followed for the overproduction of the target protein and the isolation of the crude insoluble protein fraction, which contained the TCH4 polypeptide. The insoluble proteins were separated on a 12% SDS–polyacrylamide gel. After electrophoresis, the gel was stained with aqueous 0.05% Coomassie Brilliant Blue R 250 for 10 min and washed with numerous changes of water over several hours. When the band corresponding to the TCH4 polypeptide was visible, it was excised from the gel, placed on dry ice, and sent to Cocalico Biologicals (Reamstown, PA) for innoculation into rabbits. Serum was stored at −80°C. The preimmune and anti-TCH4 antisera were affinity purified using AminoLink columns (Pierce, Rockford, IL).

Small pieces of roots and epicotyls (~2 to 4 mm^2) were fixed in a solution of 4% paraformaldehyde and 0.5% glutaraldehyde in 50 mM sodium phosphate buffer, pH 7.3, for 2 hr at room temperature before incubation overnight (12 to 14 hr) at 4°C. After fixation, the tissue was washed for 1 hr in buffer, dehydrated in a graded series of ethyl alcohol, and embedded in Steedman's wax (Norenburg and Barrett, 1987). Sections of 6 to 14 μm were cut and then affixed to the slides precoated with a high molecular mass poly-L-lysine.

The embedding medium was removed from sections with ethyl alcohol. After rehydration, sections were incubated in PBS for 12 hr before incubation in blocking solution A (2% dry milk, 0.15 M NaCl, and 0.1 M NH_4Cl in PBS). Primary anti-TCH4 antiserum was affinity purified using AminoLink columns and used at 6 μg/mL in solution A. Antibody was applied for 2 hr at room temperature in a moist chamber followed by an overnight incubation at 4°C. One 10-min rinse in solution A was followed by two 10-min washes in solution B (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 5% nonfat dry milk, 0.5% Tween 20). Secondary antibody (alkaline phosphatase–linked goat anti–rabbit IgG; Sigma) was diluted 1:100 with solution B and applied for 1 hr. Sections were then washed for 10 min in solution B, followed by two more washes in solution B without dry milk. To detect alkaline phosphatase activity, the sections were incubated in 0.165 mg/mL nitro blue tetrazolium and 0.08 mg/mL 5-bromo-4-chloro-3-indolyl phosphate in 0.1 M NaCl, 0.005 M $MgCl_2$, 0.1 M Tris-HCl, pH 9.5. After a 2-hr incubation at room temperature, the reaction was stopped by washing in 0.01 M Tris-HCl, pH 8.0, and 1 mM EDTA. Sections were mounted and then examined and photographed with the Photo Microscope III (Zeiss, Oberkoken, Germany) using differential interference contrast to visualize unstained tissue. Controls included omission of primary antibody, omission of both primary and secondary antibodies, and use of preimmune rabbit serum (affinity purified as described for the antiserum). Under the control conditions, little or no background labeling was detected.

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